IV. RESULTS AND DISCUSSION

A. INHIBITION TO HCT-116 COLON CANCER CELL

After 4 x 24 hr incubation in the presence and absence of the cell free supernatant contained SCFA, observation was conducted using inverted microscope with magnification (4x10). Cellular debris in the media could be observed on HCT-116 cells that were treated with SCFA containing 0.625, 1.00, and 1.25 mM butyrate (figure 3). This research showed that the attached cells of control became multi layer. The treated cells with 1.00 mM butyrate showed higher confluency than the treated cells with 1.25 mM butyrate, but lower than the treated cells with 0.625 mM butyrate. This results implied that higher concentration of butyrate showed higher inhibition to HCT-116 colon cancer cell. The inhibition effect was implied at butyrate concentration as low as 0.625 mM and was lower than those reported by Purwani, et. al (2010) that the inhibition effect was observed at butyrate concentration as low as 2.6 mM. SCFA derived from fermentation of RS3 of sago decreased the viability, the total cell number, and increased the inhibition growth of HCT-116 colon cancer cell in a dose dependent manner (p>0.05).

Other research concluded that butyrate inhibited Caco-2 cell proliferation at concentration low as 0.1 mM (Ruemmele, et. al 1999). This research implied inhibition at higher concentration of butyrate. This difference might caused by cancer cell difference that was used in each research, therefore difference in effect may occur.

Figure 3 Morphological change of untreated or treated HCT-116 with cell free supernatant contained SCFA. (1) untreated cells; (2) cells treated with 0.625 mM; (3) cells treated with 1.00 mM; (4) cells treated with 1.25 mM
B. DETECTION OF APOPTOSIS USING DNA LADDER ASSAY

DNA fragmentation of HCT-116 colon cancer cell was observed with DNA ladder assay using electrophoresis gel. Observation data showed different DNA bands between control and cells treated with SCFA (figure 4). DNA bands from attached cells treated with SCFA containing 0.625 mM butyrate was not significantly different from the control. However, the attached cells treated with SCFA containing 1.00 and 1.25 mM butyrate had DNA bands that were significantly different from the control: appeared as longer smear.

This research also observed DNA fragmentation of the floating cells in the medium. There were different result of DNA bands of the floating cells in the control medium and in the treated medium. There were thin DNA bands in the floating cells in the treated medium (SCFA containing 0.625, 1.00, and 1.25 mM butyrate) at ±100 bp (compared with commercial ladder), lower density than normal DNA bands. These bands were not presence in the control. This result showed that DNA of floating cells in the treated medium was fragmented and had lower density than control, the bands observed at 180-200 bp.

The appearance of the nucleosomal DNA ladder in agarose gels has become the hallmark of programmed cell death via apoptosis. This research implied that SCFA derived from fermentation type-3 resistant starch of Ipomoea batatas induced apoptosis via DNA fragmentation. DNA fragmentation was not clear in the pellet of attached cells, but was observed as a separate bands in the pellet of floating cells in the medium. Floating cells in the medium contained dead cells. This imply that SCFA induced apoptosis at very early stage in the attached cells and later stage in the floating cells in the medium which contained of dead cells.

Other researchers had reported that butyrate inhibition towards cancer cells are via apoptosis (Purwani and Suhartono 2010; Ruemmele, et. al 1999; Hinnebusch, et. al 2002). Many studies investigated the mechanism of how SCFA induced apoptosis. Purwani and Suhartono (2010) studies showed that SCFA in bacterial supernatant induced apoptosis and changed the expression of apoptosis related genes such as Bcl-2 and Bax. It also showed that bacterial supernatant consisted of SCFA increased the expression ratio of pro-apoptotic genes.

Other research concluded that SCFA especially butyrate effects are mainly due to its regulation of gene expression. That research simultaneously assessed the expression levels of 400 human genes in response to butyrate in the human colonic epithelial cell line, HT29, and found that over 10% were differentially expressed (compared to the controls) (Daly and Shirazi 2006). Studies using HeLa cells which have been exposed to sodium butyrate showed the level of histone acetylation was greatly increased as compared to control cells. The experiments indicate that butyrate inhibited activity of histone deacetylase, which influenced of the chromatin structure on the nucleosomal level (Bofa, et. al 1978).
Figure 4: DNA fragmentation of untreated and treated HCT-116 colon cancer cells

Lane Identification
1: Floating cells in the control medium
2: Floating cells in the treated medium with SCFA containing 0.625 mM butyrate
3: Floating cells in the treated medium with SCFA containing 1.00 mM butyrate
4: Floating cells in the treated medium with SCFA containing 1.25 mM butyrate
5: Attached cells control
6: Attached cells treated with SCFA containing 0.625 mM butyrate
7: Attached cells treated with SCFA containing 1.00 mM butyrate
8: Attached cells treated with SCFA containing 1.25 mM butyrate
L: Ladder 100 bp
Hinnebusch, et. al (2002) treated HT-26 and HCT-116 colon cancer cell with physiologically relevant concentrations of various SCFA, and apoptotic effects were studied by flow cytometry. Butyrate significantly increased apoptosis, whereas the other SCFA studied did not. Hinnebusch, et. al (2002) concluded that SCFA effects on apoptosis, and interestingly, only C4 (butyrate) increased the rate of programmed cell death.

Rummele, et. al (1999) studies showed that butyrate induced apoptosis (maximum 79%) Caco-2 cell line via activation of the caspase-cascade (a key event was the proteolytic activation of caspase-3, triggering degradation of poly-(ADP-ribose) polymerase (PARP)) and the mitochondrial bcl-pathway (butyrate potently up-regulatef the expression of the pro-apoptotic protein bak, without changing Caco-2 cell bcl-2 expression). Bak is a protein that induce pore formation on mitochondrial surface, therefore releasing cytochrome-c out of mitochondrial membrane forming apoptosome that could lead to activation of caspase cascade which could induce apoptosis. The caspase play an important role in apoptosis by activating Dnases, inhibiting DNA repair enzymes and breaking down structural protein in the nucleus (Elmore 2007). In summary, apoptosis is more likely when bax protein levels are high (Wyllie, et. al 1998).

The expected result of this study was clear DNA fragmentation, and there were differences DNA fragmentation of normal and apoptosis cells. However, DNA fragmentation in this search was not clearly observed. Eventhough, variation of the concentration of agarose gel (0.8%, 1%, and 2%), voltage (75 and 100 Volt), and electrophoresis time (1 and 1.5 hours) was treated, the expected DNA fragmentation was not seen clearly. This might be related with DNA extraction methods that was not optimal, and thus affected the DNA bands in the agarose.

**Figure 5. Comparison of two methods for the preparation of apoptotic DNA fragments (Herrmann, et. al 1994)**

Lane identification:
- M: 142 bp ladder
- 1: phenol extraction method, 0 hr propidium iodide staining
- 2: phenol extraction method, 3 hr propidium iodide staining
- 3: phenol extraction method, 14 hr propidium iodide staining
- 4: NP-40 lysis extraction method, 0 hr propidium iodide staining
- 5: NP-40 lysis extraction method, 3 hr propidium iodide staining
- 6: NP-40 lysis extraction method, 14 hr propidium iodide staining
Herrmann, et. al (1994) compared the methods for isolation of apoptotic DNA fragments. The research showed that method for isolation of apoptotic DNA affected the bands of DNA fragmentation. Figure 5 shows that the NP-40 lysis method with a frequently used method for isolation of apoptotic. It shows that when NP-40 lysis method produces better DNA fragmentation (figure 5 lane 4-7) compares with phenol extraction procedures (figure 5 lane 1-3). This research used ethanol extraction procedure. The fragmentation of DNA in this research was similar not clear. This research still needs optimazion methodology of DNA extraction to get the best result of DNA fragmentation assay.